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13. ABSTRACT ***(Maximum 200 Words)***

The quantity of available eukaryotic Initiation Factor 4E (eIF4E) in cells plays a critical role in the regulation of protein synthesis. EIF4E overexpression has been found in human malignancies (Li, 1997; Nathan, 1997). Furthermore, there appears to be an association of eIF4E overexpression and clinical outcomes (Li, 1998; Nathan, 1997; Li, 2001).

The purpose of the study is to determine if high eIF4E overexpression (≥7-fold increase) in patients with node negative breast cancer is associated with a statistically significant increased risk (2.5-fold) for cancer recurrence when compared to patients with low eIF4E overexpression. The study involves 255 patients treated with definitive local therapy (i.e. breast conservation therapy or modified radical mastectomy) and offered systemic adjuvant therapy per standard of care. The cancer specimens are quantified for eIF4E overexpression by Western blot analysis. Each patient undergoes identical clinical surveillance. Initial clinical stage, eIF4E overexpression, and clinical outcomes data are coded and researchers are blinded until data analysis at the end of the study. The primary endpoint measured is breast cancer recurrence; local, regional and/or distant. The projected study accrual time is 3 years, with targeted study completion in 5 years.

To date 142 patients have been accrued, 133 patients have cancer specimens quantified for eIF4E level. In an interim analysis of 111 patients, in patients with the highest tertile of eIF4E level (>14-fold), the relative risk for cancer recurrence was 6.2 x that of the low eIF4E group (<7-fold) (p=0.026).

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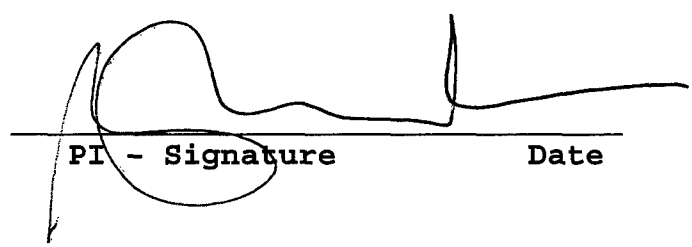

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INTRODUCTION

Eukayotic Initiation Factor 4E (eIF4E) plays an important role in the regulation of protein synthesis of mRNA with long or complex 5'UTRs (untranslated regions)[1,2,3,4]. It is elevated in breast cancer specimens, but not in normal breast tissue from non-cancer patients[5,6]. Preliminary retrospective study had suggested that the degree of eIF4E protein elevation may have prognostic significance, independent of T (size) or N (nodal) stage[7]. Given the limitations of small, retrospective studies, a prospective study of women with stage I to IIII breast carcinoma was carried out. The degree of eIF4E elevation was evaluated as an independent predictor for cancer recurrence. An interim analysis of this NCI-funded study was published and is attached as Appendix A.

As noted, high eIF4E elevation appeared to predict disease recurrence independent of nodal disease. However, neither studies were specifically designed to address whether eIF4E elevation is associated with breast cancer recurrence in node negative patients. The current project funded by the DAMD17-99-1-9256 is targeted to examine 255 patients with node negative breast cancer treated in the standard fashion (i.e. modified radical mastectomy or lumpectomy, axillary dissection, plus radiation) and offered systemic adjuvant therapy when appropriate. Tumor specimens collected at surgery are verified by the study pathologist. eIF4E levels are determined by Western blot analysis. The clinical data and outcomes of patients are collected based on a standardized follow-up protocol. The data are stored in a coded database with the researchers "blinded" until final analysis at the end of the study period. The standardized follow-up protocol includes physical examination every three months, annual mammograms, chest x-ray, and blood work (blood count and liver function tests) for years 0 to 3 after treatment, and biannual examinations thereafter. Any abnormalities will be examined and tissue diagnosis for recurrence will be documented. The endpoint of the study is to detect cancer recurrence, local (e.g. same breast), regional (e.g. same axilla), and/or distant metastasis. The intent of this study is to detect a statistically significant 2.5-fold increase in risk for cancer recurrence in patients with high eIF4E protein elevation compared to patients with low eIF4E protein elevation in a prospective study.

BODY

Task 1. Patient Accrual and Development of Data Base (months 1-36)

a. Identify and recruit 255 patients for study
b. Codify and enter pre-treatment clinical data
c. Treat breast cancer in accordance with standard of care
d. Codify, perform and record T stage, ER/PR status, and treatment received
e. Periodic quality review (every 3 months) of clinical data base
f. Integrate patients into our follow-up network to increase compliance with the study protocol

Update on Task 1

One hundred and eighty-one (181) patients with node negative breast cancer were accrued. One hundred and forty-two (142) of these patients meet all of the study inclusion and exclusion criteria. Thirty-nine were withdrawn, the majority for lack of adequate specimens for eIF4E assay. The target sample size is 255 patients. Patient accrual rate amongst eligible patients continues at >90% at LSU Heath Sciences Center-Shreveport, and at about 80% at the E.A. Conway Hospital. Pre-operative clinical data, pathologic staging, and type of treatment have been collected and updated every three months. The data have been coded and stored in a secured database.

Task 2. Quantification of eIF4E Level (Months 1-36)

a. Extract protein from cancer specimens stored in liquid nitrogen
b. Perform Western Blot analysis in triplicates
c. Quantify eIF4E levels in cancer specimens relative to
    1. normal controls (protein extract from breast tissues of non-cancer patients)
    2. known quantities of affinity column purified eIF4E protein.
d. Record eIF4E levels of individual specimens in coded data base
e. Periodic review of eIF4E assay to ensure reproducibility and accuracy (every 3 months)

Update on Task 2

A more detailed discussion of the Western blots analysis used in the quantification of eIF4E in our cancer specimens had been provided in past annual report. To date eIF4E level has been obtained in 133 cancer specimens. The pertinent characteristics of the eIF4E level and distribution are listed in the table below:

eIF4E elevation	**<7-fold**	**≥7-fold**
Number of specimens	43	90
Mean±standard deviation (eIF4E level)	4.4±1.5	14.5±5.8

As shown, of the 133 specimens tested, 43 specimens were noted to have eIF4E elevation less than 7-fold. 90 cancer specimens have eIF4E elevation ≥ 7-fold. Of those

specimens with less than 7-fold elevation, the mean degree of protein elevation is 4.4±1.5 fold (mean±standard deviation). Of the specimens that were ≥7-fold elevated, the mean degree of elevation was 14.5±5.8-fold. The proportion and degree of high versus low eIF4E elevation in cancer specimens are consistent with that reported in previous studies[5,7] .

Task 3. Surveillance Examination to Detect Recurrence (months 1-72)

a. Clinic visits by study patients per study calendar (Addendum H), i.e.
   1. every 3 months (q 3 months) year 0-3
   2. every 6 months years 4 and 5
   3. annually thereafter
b. Clinical data base to be collected on follow-up to include:
   1. symptoms and/or findings that are noted on complete history and physical examination
   2. annual mammography
   3. annual chest x-ray
   4. annual blood work (complete blood work and liver function test)
   5. any additional imaging studies to verify symptoms or abnormal findings
   6. any histology/cytology of biopsies performed
c. The primary endpoint to be measured is cancer recurrence (see definition in proposal) – all suspicious lesions identified by imaging or at clinic visits must be confirmed histologically (biopsy) or cytologically (needle aspirate)
d. Periodic review by data coordinator and PI to ensure compliance by study patients and investigators to study protocol (every 3 months)

Update on Task 3

Clinical follow-up data obtained per protocol, including physical examination, blood work, and radiologic imaging where applicable, have been updated for all 142 participants of the study. There have been 39 withdrawals from the study. Reasons for withdrawal are:

1) 20 patients with inadequate specimens for eIF4E assay
2) 6 specimens represented ipsilateral breast recurrence after initial BCT
3) 9 were inconclusively staged
4) 4 declined definitive therapy

There has not been any study associated complications.

Task 4. Interim Analysis (months 24)

a. Statistical analysis of clinical and molecular data accrued to date
b. Interim summary report to be written for DOD-sponsored meeting

Update on Task 4

Interim analysis was performed in March of this year. This was submitted as an abstract titled "eIF4E Level in Node Negative Breast Cancer – An Interim Analysis" to the Era of Hope 2002 Dept. of Defense Breast Cancer Research Program Meeting. The abstract is enclosed as Appendix B.

Task 5. Final analysis and report writing (month 72-75)

a. Final analysis of clinical and molecular data to determine if high eIF4E overexpression predicts a statistically significant increase in cancer recurrence in node negative patients
b. Manuscript preparation

Update on Task 5

This is the third year of the study. As such, final analysis will not be performed until target accrual and median follow-up have been achieved.

KEY RESEARCH ACCOMPLISHMENTS

- Patient accrual is progressing at the targeted pace.
  - >90% participation at LSUHSC-Shreveport
  - >80% at E. A. Conway Hospital with correction underway

- Interim analysis
  - Abstract submitted to Era of Hope Meeting (Appendix B)
  - Patients with the highest tertile of eIF4E elevation had an unadjusted relative risk for cancer recurrence of 6.1X that of the low eIF4E group

REPORTABLE OUTCOMES

See Appendix B

CONCLUSIONS

None to date

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APPENDICES (attached)

Appendix A
Li, B.D.L., Gruner, J.S., Abreo, F., Johnson, L.W., Yu, H., Nawas, S., McDonald, J.C., DeBenedetti, A. Prospective Study of eIF4E Protein Elevation and Breast Cancer Outcome.
Ann. Surg. 235(5)732-739, 2002.

Appendix B
Norton, K.S., DeBennedetti, A., Yu, H., Meschonat, C., Gauthier, M., Li, B.D.L. EIF4E Level in Node Negative Breast Cancer – An Interim Analysis. (submitted 2002)

Appendix A

ANNALS OF SURGERY
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Prospective Study of Eukaryotic Initiation Factor 4E Protein Elevation and Breast Cancer Outcome

Benjamin D. L. Li, MD,* Jeffrey S. Gruner, MD,* Fleurette Abreo, MD,† Lester W. Johnson, MD,§ Herbert Yu, MD,# Soheir Nawas, MD,¶ John C. McDonald, MD,* and Arrigo DeBenedetti, PhD‡

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Objective

To validate the authors' initial hypothesis-generating observation that eukaryotic initiation factor 4E (eIF4E) protein elevation predicts a higher cancer recurrence rate in patients with stage 1 to 3 breast cancer.

Summary Background Data

Tumor size and nodal status continue to be the two most important independent prognostic markers in breast cancer, despite well-documented limitations. In a previous smaller retrospective study, eIF4E, important in the regulation of protein synthesis of mRNAs with long or complex 5' untranslated regions, appeared promising as an independent predictor of breast cancer recurrence.

Methods

Specimens and clinical data from 191 patients with stage 1 to 3 breast cancer were accrued prospectively. Data collected include stage of disease, tumor grade, age at diagnosis, and menopausal status. Endpoints measured were disease recurrence and cancer-related death. eIF4E protein level was quantified using Western blot analysis. Immunohistochemical staining was used to determine estrogen receptor, progesterone receptor, and HER-2/*neu* receptor status. Statistical analysis include Cox proportional hazards model, log-rank test, Kaplan-Meier survival curve, Fisher exact test, and *t* test.

Results

Patients were divided into three groups based on tertile distribution of eIF4E: low, defined as less than 7.5-fold elevation (n = 64); intermediate, defined as 7.5- to 14-fold elevation (n = 61); and high, defined as more than 14-fold elevation (n = 66). The relative risk for cancer recurrence with intermediate elevation was 4.1 times that of patients with low elevation. For patients with high elevation, the relative risk for recurrence was higher, at 7.2 times that of the low group. The relative risk for cancer-related death for high elevation was 7.3 times that of patients with low eIF4E. Using multivariate analysis, high eIF4E remained an independent predictor of cancer recurrence after adjusting for tumor size, tumor grade, nodal disease, estrogen receptor status, progesterone receptor status, and menopausal status.

Conclusions

High eIF4E is an independent predictor of cancer recurrence in patients with stage 1 to 3 breast cancer. The relative risk for cancer recurrence increases with eIF4E protein elevation. High eIF4E elevation is also associated with an increased relative risk for cancer-related death.

To date, the two most important independent prognostic markers in breast cancer continue to be tumor size and nodal status.[1,2] However, systemic failure can and does occur in patients with small, node-negative tumors.[3] Approximately two thirds of invasive breast carcinomas present without evidence of axillary lymph node involvement, but at least 25% of these carcinomas eventually recur.[1,3] Conversely, more than 30% of node-positive breast carcinomas do not develop recurrent disease.[1] These inherent deficiencies in the TNM staging system have necessitated the search for a prognostic marker in breast carcinoma, independent of tumor size and nodal status.

Malignant transformation is a multistep process, involv-

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ing multiple alterations in the regulation and function of normal cellular activities.[4,5] These include dysregulation of protein transcription,[6,7] signal transduction,[8] and cell-cycle regulation.[9] Alterations in the regulation of protein synthesis can have profound effects on cellular metabolism and normal cell functions and have been shown to contribute to malignant transformation.[10,11] Eukaryotic initiation factor 4E (eIF4E), a 25-kd cap-binding protein involved in the initiation of protein synthesis, is one such potential molecular marker of cancer progression.[12–14]

eIF4E is a subunit of eIF4F, the purported "RNA helicase" complex that unwinds mRNAs with long 5'-untranslated regions (5' UTRs).[4–6] Specifically, eIF4E binds to the 7-methyl guanosine molecule (m7GpppN) of mRNAs, facilitating the attachment of eIF4F and the subsequent binding by the 40s ribosomal subunit. This allows for the initiation of protein synthesis.[4,5]

The 5' UTRs of the majority of mRNAs (90% or greater) are short (<200 nucleotides).[4] Thus, mRNAs with long 5' UTRs account for only about 10% of all mRNAs. Under normal cellular conditions, mRNAs with short 5' UTRs, and thus less steric hindrance, outcompete mRNAs with long 5' UTRs for protein synthesis. Indeed, mRNAs with long 5' UTRs are in effect "translationally repressed."[14,15] The binding of eIF4E to mRNAs with long 5' UTRs increases the likelihood of binding by the RNA helicase (eIF4F) and subsequent initiation of translation. eIF4E is the rate-limiting component in this binding reaction.[7,16] Thus, an abundance or overexpression of eIF4E leads to an increased likelihood that mRNAs with long 5' UTRs will be translated.[4]

Some of the gene products upregulated by eIF4E overexpression include cyclin D1[10] and c-myc,[17] important in cell-cycle regulation; ornithine decarboxylase,[11] important in bioamine synthesis; and two angiogenic factors, basic fibroblast growth factor (FGF-2)[18] and vascular endothelial growth factor (VEGF).[19] In addition, a mammalian Tousled-like kinase (TLK1B) has been reported to be upregulated by eIF4E overexpresssion.[20] This results in the phosphorylation of histone H3 and confers radioresistance to transfected cells.

In vitro models have shown that transfection of cell lines such as Chinese hamster ovaries (CHO), HeLa, and cloned rat embryo fibroblasts (CREF) by viral vectors with eIF4E overexpression result in the acquisition of malignant phenotype.[12,16,17] This malignant phenotype is manifested by loss of contact inhibition, acquisition of anchorage-independent growth, and increased cell division. Conversely, when eIF4E overexpression is inhibited using antisense oligonucleotides to eIF4E, the acquired malignant phenotype is reversed.[16,21]

In addition, several malignant breast cell lines have been shown to have eIF4E overexpression.[13] Kerekatte et al[22] first reported that eIF4E was overexpressed in human breast carcinoma but not in benign breast specimens. We reported in a small retrospective study that the degree of eIF4E protein elevation may have potential as a molecular prognostic marker for breast cancer recurrence.[23] In that study, eIF4E protein levels from cancer specimens were quantified using Western blots in 59 patients with stage 1 to 3 breast carcinoma. In the 38 patients with high eIF4E overexpression (defined as sevenfold elevation or more), 14 patients had breast cancer recurrences ($P = .03$, log-rank test), of whom 11 died from the disease ($P = .04$, log-rank test). In the group with low eIF4E overexpression (defined as less than sevenfold), one cancer recurrence was detected.

However, inherent biases in small, retrospective studies are well documented.[24,25] Thus, to verify and confirm our initial observations, as well as to test our hypothesis that eIF4E protein elevation predicts a worse clinical outcome in patients with stage 1 to 3 breast carcinoma, we designed a study with the following objectives: to prospectively accrue patients with stage 1 to 3 breast cancer, to quantify the eIF4E level in cancer specimens, to ensure compliance with a standardized breast cancer treatment protocol, to maintain a standardized clinical follow-up protocol to detect cancer recurrence and cancer-related death, and to determine whether a high eIF4E level portends a worse clinical outcome.

METHODS

One hundred ninety-one patients with stage 1 to 3 breast cancer were accrued prospectively in this institutional review board-approved study. All patients were treated with definitive surgical therapy (i.e., modified radical mastectomy or breast conservation therapy and adjuvant radiation therapy) with curative intent. Systemic adjuvant therapy (chemotherapy and/or hormonal therapy) was offered as per standard of care for the stage of disease. A standardized posttreatment clinical follow-up protocol was strictly adhered to: history and physical examinations every 3 months for the first 3 years, then every 6 months thereafter until year 5, then annual visits for the lifetime of the patient. In addition, annual mammograms, blood work (complete blood count and liver function tests), and chest radiographs were performed. Additional imaging and other diagnostic tests were directed by abnormal findings. The primary endpoints measured were disease recurrence (local, regional, systemic) and cancer-related deaths.

From each patient, at the time of primary surgical intervention, a fresh piece of tumor specimen of at least 100 mg was obtained. This was identified and verified by the study pathologist (F.A.). The specimen was immediately frozen in liquid nitrogen and stored at −70°C. A code was given to each specimen at the time of collection for tracking purposes. One set of note cards, accessible only to the principal investigator, linked the specimen code with the patient data. To avoid inadvertent bias, the study pathologist, the investigators involved in the assay for eIF4E and other tumor markers, and the data coordinator were unaware of patients' data sets.

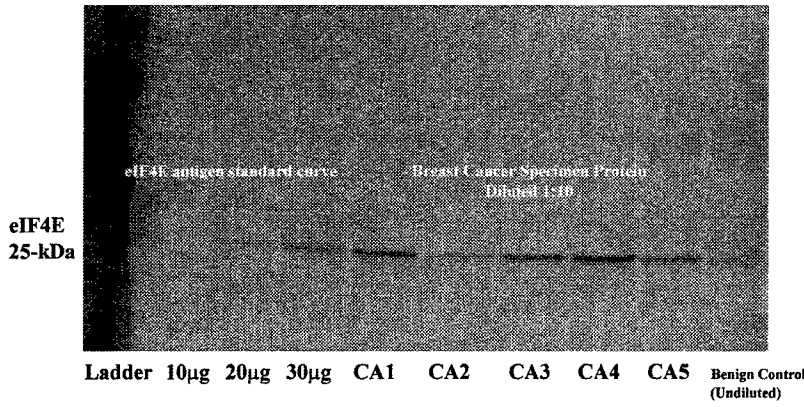

Figure 1. A typical Western blot showing the intensity of the 25-kd eIF4E protein band in carcinoma specimens CA1 through CA5 relative to benign control and the eIF4E standard curve.

Specimen assay for eIF4E protein level was performed using Western blot analysis, a technique previously described in detail.[22,23] Briefly, protein lysate from each breast specimen was prepared using 10 mg tissue cut into tiny pieces, suspended in 0.5 mL RIPA buffer (150 mmol/L NaCl; 1% NP-40; 0.5% DOC; 0.1% SDS; 50 mmol/L Tris [pH 8.0]; 0.1 mmol/L PMSF) and mechanically macerated using a Savant Bio 101 Fastprep FP120 system (Savant Instruments, Inc., Holbrook, NY). The macerate was then centrifuged (10,000*g* for 10 minutes at 4°C), and the concentration of protein suspension was determined using the BCA protein assay kit (Pierce, Rockford, IL) against a standard graph of known BSA protein concentrations.

Equal amounts of protein lysate from each breast specimen (20 μg diluted in 1:10 RIPA) were separated using 4% to 20% denaturing gel Tris-HCl polyacrylamide gel electrophoresis. The proteins were then electroblotted onto a nylon membrane (Immobilon PVDF, Millipore, Bedford, MA), and the membranes were blocked in 5% nonfat milk for 1 hour. Primary incubation of the membrane was then carried out using a 1:500 dilution monoclonal mouse anti-eIF4E antibody (Transduction Laboratories, San Diego, CA). Secondary incubation of the membrane was then carried out using a 1:1,000 dilution of goat-antimouse horseradish peroxidase conjugate. The blot was then developed using Opti 4CN (4-chloro-1-naphthol, Bio-Rad Laboratories, Hercules, CA). The blots were scanned using the Biophotonics system (Biophotonics Corp., Ann Arbor, MI) and band intensity was evaluated using Intelligent Quantifier software (Bio Image, Ann Arbor, MI).

The quantification of eIF4E levels in each cancer specimen was performed using two standards. The degree of eIF4E elevation is expressed as the number of folds over protein lysate controls of breast specimens from patients without cancer. In addition, on the same blot, a standard curve of a known concentration of eIF4E protein was used to internally verify the quantification process (Fig. 1). Triplicates of each specimen were run and the results averaged.

Immunohistochemical staining for estrogen receptor (ER), progesterone receptor (PR), and HER-2/*neu* was performed on a Dako Autostainer (Dako Corp., Carpinteria, CA) using standard protocols. Slides were evaluated using the Automated Cellular Imaging System (Chromavision Medical Systems, Inc., San Juan Capistrano, CA). ER and PR results were reported based on the degree and intensity of nuclear staining for all receptors respectively, with a positive result defined as greater than 10% staining. HER-2/*neu* slides were evaluated using the Hercep program (Dako Corp., Carpinteria, CA), with a positive result defined as 2 or more.

Survival and disease-free survival analysis were performed using the Kaplan-Meier method and the log-rank test. The Fisher exact test was used to determine eIF4E protein elevation in relation to other known prognostic markers. The Cox proportional hazard model was used to test for eIF4E as an independent prognostic marker and to determine the relative risk for cancer recurrence and cancer-related death.

RESULTS

The clinical data and the pathologic features of the specimens from the 191 patients with stage 1 to 3 breast cancer are summarized in Table 1. The median age was 52 years. Forty-nine patients underwent breast conservation therapy and 142 underwent modified radical mastectomies. Eleven percent of patients had T3 lesions, 45.5% had T2 lesions, and 43.5% had T1 lesions. Most tumors were grade 2 or higher, and 46.6% had nodal involvement. For patients whose specimen receptor status was available, 48.7% were ER positive, 40% were PR positive, and 30.3% were HER-2/*neu* positive. The median follow-up of the study is 19 months. To date, 27 patients have had cancer recurrences, 15 of whom have died from the disease.

eIF4E protein level was determined using Western blots. As in previous studies, eIF4E protein level in cancer specimens was quantified relative to benign specimen controls from patients without cancer as the number of folds over the baseline eIF4E level. A typical Western blot for eIF4E overexpression is shown in Figure 1. eIF4E overexpression is readily shown by the higher intensity of the eIF4E band in the carcinoma specimens (lanes CA-1 to CA-5), even after a 1:10 dilution of the protein lysate. In contrast, undiluted protein lysate from a benign specimen of a patient

Table 1. PATIENT DEMOGRAPHICS

Characteristic	No. of Patients (%)
Age, years	
< 50 years	74 (38.7)
≥ 50 years	117 (61.3)
Median = 52 years	
Range = 27–90 years	
Surgical treatment	
Breast conservation	49 (26)
Modified radical mastectomy	142 (74)
T stage	
T1	83 (43.5)
T2	87 (45.5)
T3	21 (11.0)
Node status	
Positive	89 (46.6)
Negative	102 (53.4)
Grade	
1	8 (5.3)
2	96 (63.2)
3	48 (31.5)
Estrogen receptor status	
Positive	75 (48.7)
Negative	79 (51.3)
Progesterone receptor status	
Positive	60 (40)
Negative	90 (60)
HER-2/*neu* status	
Positive	37 (30.3)
Negative	85 (69.7)
Adjuvant therapy	
Chemotherapy	106 (55.5)
Hormonal therapy (tamoxifen)	100 (52.4)
Relapses	27
Deaths	15
Duration follow-up, months	
Median = 19 months	
Range = 1–125 months	

without cancer is shown in the last lane. This band is used as a baseline control for the quantification of eIF4E elevation. An eIF4E antigen standard curve is also shown (lanes 2–4).

In the cancer specimens tested, eIF4E protein was elevated between 1.9-fold and 48.4-fold over benign controls. The mean (± SD) elevation of eIF4E protein in cancer specimens was 12.2 ± 7.8-fold. Based on the degree of eIF4E elevation, patients were distributed into three similarly proportioned groups or tertiles. In group 1, 64 patients had low eIF4E elevation (<7.5-fold overexpression). In group 2, 61 patients had intermediate eIF4E levels (7.5- to 14-fold elevation). In group 3, 66 patients had high eIF4E elevation (>14-fold overexpression). The tertile distribution of patients based on eIF4E protein elevation is represented in a pie chart in Figure 2.

Elevation of the eIF4E protein level was compared with other known potential prognostic markers in breast carcinoma. Using the Fisher exact test, eIF4E level was analyzed relative to tumor size, tumor grade, nodal disease, ER status,

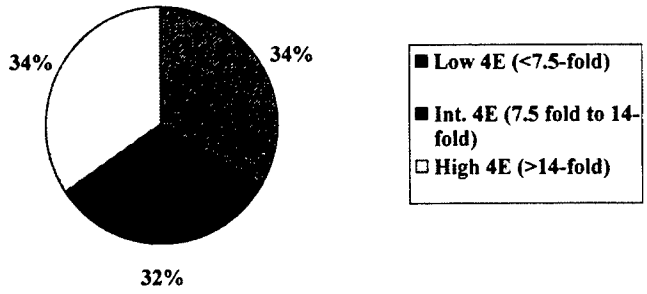

Figure 2. Tertile distribution of eIF4E overexpression.

PR status, HER-2/*neu* receptor status, and menopausal status. As shown in Table 2, high eIF4E level was associated with high tumor grade (P = .019), PR-positive disease (P = .027), and nodal disease (P = .028).

In the low eIF4E group, there were three recurrences (two systemic, one locoregional and systemic) and two deaths. In the intermediate group, there were eight recurrences (three locoregional, four systemic, and one both) and five deaths. In the high group, there were 16 recurrences (4 locoregional, 11 systemic, and 1 both) and 8 deaths.

Breast cancer recurrence was analyzed using the Kaplan-Meier method. Comparing the three groups of patients with stage 1 to 3 breast cancer, the high group had the worst disease-free survival (Fig. 3). This was statistically significant (P < .001, log-rank test). The intermediate group fared worse than the low group but better than the high group.

When cancer-related death was analyzed using the Kaplan-Meier method, the high group fared the worst (Fig. 4). The intermediate group fared in between the high and the low group. This was also statistically significant (P = .003, log-rank test).

Multivariate Cox proportional hazard analysis was performed where nodal status, tumor size, tumor grade, ER status, and PR status were considered together with eIF4E protein elevation in predicting cancer recurrence. As shown in Table 3, nodal status (P = .01) and high eIF4E protein overexpression (P = .019) were independent predictors of disease recurrence.

The relative risk for cancer recurrence for high and intermediate eIF4E protein elevation versus low eIF4E was calculated. The intermediate group had 4.1 times the risk for

Table 2. eIF4E AND CLINICOPATHOLOGIC FEATURES

	P Value (Fisher exact test)
Tumor grade	.019
Progesterone receptor status	.027
Nodal status	.028
Estrogen receptor status	.108
Tumor size	.205
HER-2/*neu* status	.426
Menopausal status	.898

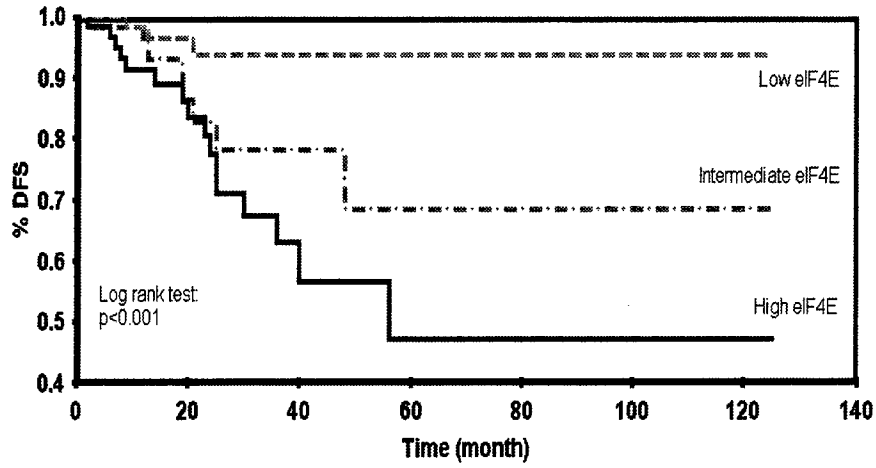

Figure 3. Comparison of disease-free survival using the Kaplan-Meier method between the groups of patients with low, intermediate, and high eIF4E protein elevation.

cancer recurrence compared with the low group (P = .037, 95% confidence interval [CI], 1.09–15.6)) (Table 4). In the high group, the relative risk for cancer recurrence was 7.2 times that of the low group (P = .002, CI 2.08–24.8).

The relative risk for cancer-related death was also examined. The intermediate group had a relative risk of 4.1 times that of the low group. This has not reached statistical significance yet (P = .091, CI 0.797–21.4) (Table 5). In the high group, the relative risk for cancer-related death was 7.3 times that of the low group. This was statistically significant (P = .011, CI 1.58–33.9).

DISCUSSION

This study was undertaken to validate our initial observation that high eIF4E protein overexpression in cancer specimens of patients with stage 1 to 3 breast cancer had a significantly higher rate of cancer recurrence and cancer-related death. The initial study was small and retrospective; as such, inherent biases may have included variability in treatment compliance, follow-up for disease recurrence, and false-positive results. Thus, this study was designed with the following objectives: to prospectively accrue patients with stage 1 to 3 breast cancer, to quantify cancer specimen eIF4E level, to ensure patient compliance with a standardized breast cancer treatment protocol, to maintain a standardized clinical follow-up protocol to detect cancer recurrence and cancer-related death, and to determine whether a high eIF4E level predicts a worse clinical outcome.

One hundred ninety-one patients with stage 1 to 3 breast cancer were accrued. All of the patients were treated with standard surgery (breast conservation therapy with radiation therapy or modified radical mastectomy) and adjuvant therapy. A standardized clinical follow-up protocol to detect recurrent disease was strictly adhered to. Compliance with treatment and follow-up exceeded 95%.

The patients were distributed into tertiles based on eIF4E protein elevation: low eIF4E elevation (<7.5-fold, n = 64 patients), intermediate eIF4E elevation (7.5- to 14-fold, n = 61), and high eIF4E elevation (>14-fold, n = 66). The relative risk for cancer recurrence in the intermediate group was 4.1 times that of the low group (P = .037). For the high group, the relative risk for cancer recurrence was even higher, at 7.2 times that of the low group (P = .002). The relative risk for cancer-related death for the high group was 7.3 times that of the low group (P = .01).

The clinical follow-up for this study is short: the median follow-up is 19 months. Nonetheless, the patients with high eIF4E levels have consistently shown a significantly higher

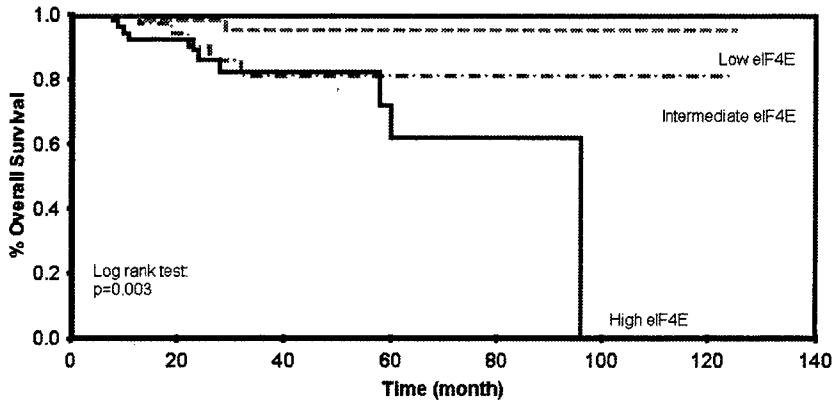

Figure 4. Comparison of cancer-related death rates using the Kaplan-Meier method between the groups of patients with low, intermediate, and high eIF4E protein elevation.

Table 3. COX MULTIVARIATE ANALYSIS FOR DISEASE RECURRENCE

	95% Confidence Interval	*P* Value
High eIF4E overexpression	1.533–119.8	.019
Nodal status	1.859–91.07	.010
Menopausal status	0.8040–11.45	NS
Tumor size	0.2994–1.796	NS
Tumor grade	0.6414–3.932	NS
Estrogen receptor status	0.3403–8.458	NS
Progesterone receptor status	0.1718–5.238	NS

relative risk for cancer recurrence and cancer-related death. In the intermediate group, there is a greater relative risk for cancer recurrence, but not cancer-related death yet.

eIF4E elevation as a marker for cancer recurrence has been reported in other solid tumors. Nathan et al[26] reported that eIF4E elevation in the tumor-free margins of squamous cell carcinoma of the head and neck after curative resection was associated with an increased cancer recurrence rate. In that study, patients with eIF4E elevation detected in the tumor-free surgical margins had a cancer recurrence rate of 56%. In contrast, when eIF4E elevation was absent, the recurrence rate was 6.9% On multivariate analysis, eIF4E elevation at the margins persisted as an independent prognostic marker for cancer recurrence ($P = .009$).

Efforts at identifying prognostic markers in breast cancer independent of TNM stage have met with mixed results. When eIF4E level was analyzed relative to tumor size, tumor grade, nodal status, ER status, PR status, HER-2/*neu* status, and menopausal status, high eIF4E level was associated with high tumor grade ($P = .019$), PR-positive tumor ($P = .027$), and node-positive disease ($P = .028$). In addition, on multivariate analysis, after adjusting for ER status, PR status, tumor size, tumor grade, nodal status, and menopausal status, high eIF4E elevation continues to be a significant independent prognostic marker for cancer recurrence.

The recent interest in the importance of hematologic dissemination, as represented by the detection of micrometastasis in bone marrow aspirate in predicting cancer outcomes, may represent a shift in paradigm in the way we conceptualize staging of breast cancer.[27] In light of the reported association of eIF4E overexpression and the upregulation of two potent angiogenic factors (VEGF and FGF-2) in vitro[18,19] and the increased relative risk for cancer recurrence in patients with high eIF4E that is independent of nodal disease, the potential of eIF4E as a marker for hematologic dissemination is especially intriguing.

Table 4. RELATIVE RISK: eIF4E AND CANCER RECURRENCE

	Relative Risk	95% Confidence Interval	*P* Value
Intermediate eIF4E overexpression*	4.1	1.091–15.634	.037
High eIF4E overexpression*	7.2	2.083–24.790	.002

* Compared with low eIF4E overexpression.

Table 5. RELATIVE RISK: eIF4E AND CANCER-RELATED DEATH

	Relative Risk	95% Confidence Interval	*P* Value
Intermediate eIF4E overexpression*	4.1	0.797–21.423	.091
High eIF4E overexpression*	7.3	1.587–33.865	.011

* Compared with low eIF4E overexpression.

In our laboratory, we have preliminary data showing that eIF4E overexpression is associated with the upregulation of VEGF in human breast cancer specimens (manuscript in preparation). In addition, VEGF and FGF-2 upregulation has been associated with increased microvessel density.[28–30] Further, an increase in microvessel density and a worse clinical outcome for breast cancer has been observed.[31,32] A potential linkage of high eIF4E overexpression, resulting in angiogenic factors upregulation, subsequent increase in tumor microvessel density, and a worse clinical outcome may exist. Thus, the potential of eIF4E as an early marker of risk for hematogenous dissemination, independent of nodal disease, needs further investigation. Studies on this potential linkage are underway.

CONCLUSIONS

A high eIF4E level is an independent predictor of cancer recurrence in patients with stage 1 to 3 breast cancer. The relative risk for cancer recurrence increases with eIF4E protein elevation. High eIF4E elevation is also associated with an increased relative risk for cancer-related death. Therefore, eIF4E protein elevation in breast cancer specimens is associated with an increased risk for a worse clinical outcome in patients with stage 1 to 3 breast cancer.

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Discussion

DR. KIRBY I. BLAND (Birmingham, AL): This is a very timely paper. As you can see, there is current great interest in identification and characterization of biomarkers for breast cancer. Currently we include those as risk biomarkers, drug effect biomarkers, surrogate endpoint biomarkers, and, more recently, the predictive or prognostic variance for response to therapy.

There have been many papers presented before this Association that relate to cellular, biochemical, and prognostic markers in this particular organ site cancer. I would point out to you that for cellular markers, the ones that have really maintained permanence in our use of these on a daily basis for clinical care have been S phase or Ki67 rates and grade of the tumor for cellular variance, and for biochemical markers, ER/PR receptor, which correlate with survival.

Now we are in an era in which molecular markers are being consistently utilized and developed. And they include HER2/*neu*, p53, and now Dr. Li and Dr. McDonald and their associates from LSU have added a new one that is very important. In this regard, overexpression of 4E has been associated with upregulation of c-myc, cyclin D1, and VEGF. In addition, when they transfected their cell lines, this has been shown that 4E has actually induced the tumor to acquire a very malignant phenotype.

In their 191 patients, Dr. Li has found that protein expression was increased 2- to 48-fold compared to benign breast parenchyma for non-cancer patients. The authors have grouped these, as you just saw, into low, intermediate, and high overexpression of 4E and determined that high-grade PR-positive disease and node-positive disease are the correlates for expression.

So this leads to questions, because I am intrigued that high-level overexpression of 4E was a significant factor but not the intermediate and low level. It perhaps has to do with the numbers you are looking at, then, in the series and the mechanistic methods in which you are doing this. So that leads to the question, is there a mechanistic or molecular explanation for the importance of high-level versus low or intermediate overexpression of this factor?

Second, breast cancer investigators are currently focusing on the development of risk biomarkers for ER-negative, node-negative patients. The

first paper this morning by Dr. Wood actually focused on the node-negative patients and what to do with those individuals, because there are many more of those without nodal disease that we really need to make decisions about how to treat. So this leads to the question, what is the significance of overexpression of 4E in this patient group; that is, again, the ER-negative, node-negative patient group? And do you have future plans for investigations?

Finally, I would ask you about the estrogen levels in these patients. I think any future correlates should really look at the menopausal status and the absolute estrogen values, particularly in the follicular phase, days 1 through 13. Do you have any plans to look at that data?

I found this to be a most interesting paper. I am glad he brought it to the attention of the Association. I enjoyed the manuscript. And I congratulate the authors on a timely presentation.

DR. GERARD M. DOHERTY (St. Louis, MO): Congratulations on a very nice prospective validation of your previous observations. I think the fact that this is prospective is very important. I have two quick questions for you.

One, have you done immunohistochemistry to look at eIF4E 40 levels, and are these differences detectable that way? I don't think our pathologists are going to do quantitative Western blotting with the same facility that you have, for us to be able to translate this into clinical prognostic use.

The second thing is that you seem to have identified an important pathway in the aggressiveness of these breast cancers. While prognosis and biomarker information is great, what we really want is better treatments. Have you speculated about how you might use this information about the overexpression of eIF4E and maybe a competitive advantage for translation of these long terminal repeat proteins in the cancer patients? Is there some way you can inhibit that activity?

DR. BENJAMIN D. L. LI (Shreveport, LA): Thank you for the kind comments and very insightful questions.

Dr. Bland, we have analyzed 4E levels as a continuum and in discrete data sets, as in high, intermediate, and low groups. The risk for cancer recurrence as well as for cancer death increases with 4E elevation in discrete data sets under a continuum. In this study we broke it down to three proportionate groups because it is easy for data presentation and demonstrates the results in a more clear-cut fashion. In fact, if you look at our data, the high group as well as the intermediate group are statistically significant in determining cancer recurrence when compared to the low group. However, in cancer-related death, only the high group at this time has reached statistical significance compared to the low group. I think this is due to the short median follow-up in this prospective study, being only 19 months. I suspect that the intermediate group will eventually reach statistical significance, as the *P* value currently is .09.

With regards to your second question about node-negative disease, we have a specifically designed prospective study looking at node-negative breast cancer patients and clinical outcome relative to 4E elevation. That study is currently accruing patients. As you might expect, node-negative patients' recurrences will take a little longer, so I don't think we will have mature data for another 4 or 5 years.

We did look at menopausal status, and it did not predict recurrence. I did not look at the degree of ER/PR as a predictor of whether the outcome may be affected by eIF4; I think that is a wonderful idea. A lot of these ER receptor data were obtained by immunohistochemical staining.

Dr. Doherty, we have looked at immunohistochemical staining for 4E. In fact, if you look at IHC slides, and we have presented IHC data at other meetings, 4E is highly overexpressed in the infiltrating ductal carcinoma but not in the surrounding tissue. However, as you well know, quantification by immunohistochemical techniques is much more challenging. We have used a number of systems to try to quantitate IHC for 4E, and we have not had the same reproducibility with IHC as with Western blots.

Finally, how is this going to be helpful to our patients? Low 4E elevation as a prognostic marker may identify a subset of patients that do not need chemotherapy. Therapeutically, my colleagues in molecular biology have been working on a gene construct with a long 5' UTR that exploits cancer cells with overexpression of 4E. The same construct allows for a suicidal gene to be factor transfected into cancer cells. Since cancer cells overexpress 4E, the suicide gene will be preferably expressed in cancer cells.

EIF4E LEVEL IN NODE NEGATIVE BREAST CANCER – AN INTERIM ANALYSIS

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Introduction: Though tumor size and the presence of nodal disease continue to best predict breast cancer outcome, 25% or more of patients with stage I breast cancer will recur. Eukaryotic Initiation Factor 4E (eIF4E) binds to mRNAs with long 5 prime untranslated regions (5' UTRs) and regulates protein synthesis. In a prospective study, we reported that high eIF4E protein level in tumor specimens from women with stage I to III breast cancer are at higher risk for cancer recurrence (Hazard ratio = 7.2, CI 2.08 - 24.8) and cancer-related death (Hazard ratio = 7.3, CI 1.59 – 33.9). On multivariate analysis, after accounting for tumor size and nodal status, high eIF4E level continued to be an independent prognostic marker for cancer recurrence. It thus raises the critical question of whether high eIF4E level may independently predicts cancer recurrence in node negative patients. In this prospective study, our hypothesis is that high eIF4E level in tumor specimens of patients with node negative breast cancer independently predicts cancer recurrence and a worse clinical outcome.

Methods: An accrual target of 242 patients with node negative breast cancer was designed to detect a 2.5-fold increase in relative risk for cancer recurrence. In this interim study, 111 patients were available for analysis. Patients were treated with either a modified radical mastectomy or breast conservation therapy (+XRT), with or without adjuvant therapy. A standardized clinical follow-up protocol was strictly adhered to. Clinical data and eIF4E quantification of tumor specimens were performed and analyzed. End points measured were cancer recurrences and cancer-related deaths.

Results: Based on tertile distribution of eIF4E levels, the patients were divided into 1) low eIF4E (< 7.5-fold, n=43), 2) intermediate eIF4E (7.6 to 14-fold, n=37), and 3) high eIF4E (> 14-fold, n=31). The relative risk ratios for cancer recurrence and cancer-related death are shown in the table below:

Variable	Disease-free Survival		Overall Survival	
	Unadjusted RR[1] (95% CI)[3]	Adjusted[2] RR (95% CI)	Unadjusted RR (95% CI)	Adjusted RR (95% CI)
4E≤7.5	1.00	1.00	1.00	1.00
4E=7.6-14	2.00 (0.33-12.1)	1.41(0.22-8.91)	3.59 (0.32-40.7)	1.42 (0.11-18.7)
4E≥14.1	6.21 (1.19-32.6)	2.74 (0.47-16.0)	7.85 (0.85-72.4)	2.31 (0.21-26.1)
Test for trend	p=0.026	p=0.250	p=0.053	p=0.462

1. RR: Relative risk
2. Adjusted for age, tumor size, ER and PR.
3. CI: Confidence interval.

In the low eIF4E group, 3 patients have had cancer recurrences, with one cancer-related death. In the intermediate eIF4E group, 3 cancer recurrences and 2 cancer-related deaths have occurred. In the high eIF4E group, 6 cancer recurrences and 4 cancer-related deaths have occurred.

Conclusions: In this interim analysis, patients with breast cancer that are in the highest tertile of eIF4E overexpression had an unadjusted relative risk for cancer recurrence of 6.1X that of the low eIF4E group. This has reached statistical significance. Analysis of cancer-related death has not reached statistical significance, due to the ongoing nature of the study and its short follow-up.

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